



# Protecting the Nation's Livestock

*A Livermore-developed assay rapidly detects foot-and-mouth disease and look-alike livestock diseases.*

**S**INCE 2001, the U.S. has improved its ability to prevent and respond to terrorist attacks, but the nation's agricultural infrastructure could be vulnerable. Although the nation has never experienced an attack targeted at agriculture, such an incident, called agroterrorism, could have devastating consequences.

America's sheep, cows, pigs, and chickens are particularly vulnerable to diseases that are endemic in other parts of the world but have not appeared in this country for many decades. If allowed to gain a foothold in the U.S., a foreign animal disease (FAD) could spread

rapidly and be difficult to contain. It could thus severely affect millions of animals and the national economy.

Agroterrorism poses a threat because U.S. farms and feedlots are rarely guarded and widely dispersed across the country. Should an infection occur, normal livestock and personnel movement, including interactions with other herds, could lead to widespread dissemination of a disease before it is diagnosed. In addition, the clinical signs of FADs closely mimic many diseases that regularly occur in the nation. Homeland security experts are thus concerned that farmers, local veterinarians, and others will not be able

to recognize and report a FAD before it spreads out of control.

A great concern centers on foot-and-mouth disease (FMD), a highly contagious livestock disease that has not been seen in the U.S. since 1929. During the past decade, however, outbreaks of FMD in the United Kingdom, Asia, and South America have heightened awareness of the potential danger. The U.S. clearly needs the capability to identify FMD early and provide a rapid, large-scale response, including the diagnostic support for controlling the outbreak.

To address this need, a Livermore team, with support from the Department of Homeland Security (DHS) and the U.S. Department of Agriculture (USDA), has developed a rapid assay that simultaneously detects FMD and other foreign and endemic diseases with similar symptoms. The assay is being evaluated by 13 laboratories within the National Animal Health Laboratory Network (NAHLN) and by the National Veterinary Services Laboratory (NVSL) at Plum

Island, New York. Once validated and accepted by the USDA, the Livermore assay will allow laboratories to quickly diagnose FMD and diseases that mimic its symptoms.

“Our goal is to provide federal and state stakeholders with significantly improved detection, identification, and response capabilities for foreign and endemic agricultural diseases,” says Ray Lenhoff, a molecular virologist in Livermore’s Biosciences Directorate. The FMD assay development team, which is led by Lenhoff, also includes analytical chemist Ben Hindson and veterinarian Pam Hullinger of the Laboratory’s Physics and Advanced Technologies Directorate; toxicological immunologist Max Rasmussen, also in Biosciences; and other Livermore experts in homeland security, pathogens, and advanced detection methods. The team is part of the Bioassays and Signatures Program, which develops and implements methods to diagnose agents of bioterrorism. This program operates within the Laboratory’s Chemical

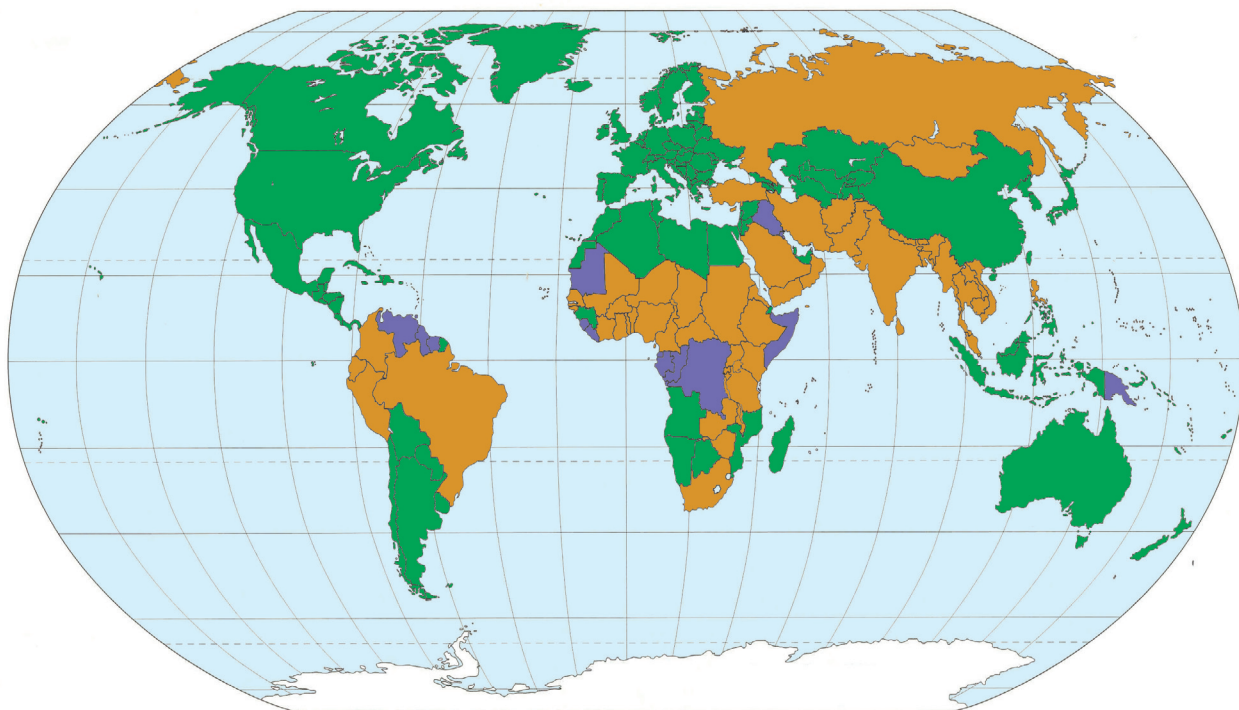
and Biological National Security Program, whose mission is to improve the nation’s capabilities to prevent, prepare for, detect, and respond to terrorist use of chemical or biowarfare agents.

### On the Front Lines

Lawrence Livermore has been on the front lines of the nation’s biodefense effort since the mid-1990s. Laboratory-developed signatures—specific regions of DNA or RNA that uniquely identify a pathogen—have been used for homeland defense since 2001. For example, Livermore assays are incorporated in DHS environmental monitoring systems such as BioWatch.

The Laboratory has teamed with state and federal departments of agriculture in the past. In 2002, Livermore, the California Animal Health and Food Safety Laboratory at the University of California at Davis, and USDA scientists worked together to contain a natural outbreak in southern California of exotic Newcastle disease, an extremely contagious and fatal viral disease affecting all species of

Foot-and-mouth disease (FMD) is endemic in many parts of the world. In this map, brown indicates countries that reported FMD occurrences in 2004, green shows those with no reported occurrences, and purple denotes those for which data are incomplete or unavailable. (Data from the World Organisation for Animal Health.)



birds. At the time of the outbreak, existing assays required 6 to 12 days to identify the disease. A key signature isolated by a team of Livermore researchers allows the virus to be identified within 4 hours.

In February 2001, during the height of the FMD outbreak in the United Kingdom, a Livermore team analyzed the virus's tiny genome of 8,000 RNA bases. The team determined that only one region of the FMD genome can support a signature-based assay. (See *S&TR*, April 2004, pp. 4–9.)

### FMD: An Enormous Threat

FMD is an extremely contagious viral disease of pigs and domesticated and wild ruminants such as cattle, sheep, goats, deer, and water buffalo. Animals afflicted with FMD usually do not die, but the disease is debilitating and animals do not recover. An infected animal begins to drool, and virus-filled blisters develop in its mouth and at the junction where skin connects to the hoof wall, making it painful for the animal to walk. In addition, the virus attacks the mammary glands, leading to reduced milk production. The vaccine for FMD only reduces the severity of symptoms; it does not prevent infection or completely stop transmission of the disease. Humans do not get sick, but they can act as carriers of the virus.

FMD is endemic in many nations and remains the most important barrier to world trade in live animals and animal products. In 2000 and 2001, a Pan-Asian strain caused outbreaks in Korea, Japan, Russia, Mongolia, South Africa, the United Kingdom, France, and the Netherlands.

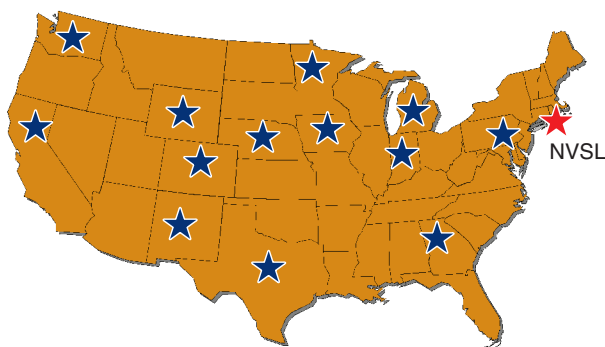
Economic losses from an agroterrorist incident, especially one caused by an extremely contagious disease such as FMD, could be enormous. (See the box on p. 16.) The potential costs include testing thousands (perhaps millions) of animals, destroying and disposing of infected animals, quarantining uninfected premises, and disinfecting and repopulating farms. In addition, such an outbreak would jeopardize

the business continuity of the domestic livestock industries, including many jobs in agriculture, food processing and production, domestic and export markets. "Early detection is critical to limiting the scope of an outbreak," says Hullinger. One estimate is that the U.S. would lose up to \$3 million in direct costs for every hour's delay in diagnosing FMD.

Currently, a veterinarian or farmer who suspects a case of FMD contacts the USDA or a state agricultural department, which sends a veterinarian who has been

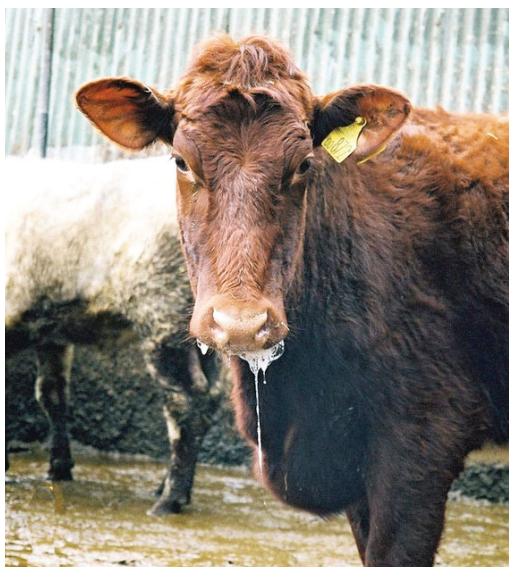
trained by the USDA to diagnose FADs. This diagnostician questions the farmer, examines the animals, and if warranted, sends specimens to the Plum Island laboratory for definitive analysis.

Plum Island averages about 300 FAD investigations a year. Many of the tests it uses, such as those to isolate the FMD virus, are time-consuming and laboratory intensive. During an FMD outbreak, the demand for analytic services would almost certainly far exceed the capacity at NVSL. "In such cases, the NAHLN



Livermore's assay for foot-and-mouth disease is being evaluated by 13 National Animal Health Laboratory Network laboratories (blue stars) and the National Veterinary Services Laboratory (NVSL) at Plum Island, New York (red star). Without a fast and accurate assay, an outbreak could rapidly overwhelm NVSL's diagnostic capabilities.

(a)



(b)



When this shorthorn heifer in the United Kingdom became afflicted with foot-and-mouth disease, (a) she began to drool, and (b) virus-filled blisters developed in her mouth and at the junction where skin connects with the hoof walls.

labs could provide regional and scalable diagnostic support under the direction of the USDA,” says Hindson. “The availability of a validated, rapid test for detecting and identifying FMD and differentiating it from other diseases that present similar clinical signs would be invaluable.”

### A Tool for Homeland Security

The Livermore animal assay research, which began in early 2005, is funded by DHS’s Agricultural Assays and Agricultural Security Domestic Demonstration and Application Program. DHS is charged with coordinating U.S. efforts to protect against agroterrorism, while the USDA is responsible for responding to major outbreaks of disease involving agriculture.

Within the USDA, the Animal and Plant Health Inspection Service (APHIS) is responsible for protecting animals and plants from agricultural pests and diseases, including the introduction of FADs, and for coordinating the response to an agricultural disease outbreak. NVSL, which is part of APHIS, coordinates the state and university veterinary diagnostic laboratories belonging to NAHLN.

In developing signatures for bacterial and viral pathogens, Livermore scientists use the KPATH technique, which was

developed by Laboratory computer scientist Tom Slezak. KPATH compares the genome of a target pathogen to the existing library of microbial genomes, searching for areas that are unique to the target organism. The DNA or RNA regions selected are present in every strain of the pathogen and absent in all other organisms sequenced to date. The assay development team also takes advantage of advances in rapid polymerase chain reaction (PCR). This technique makes multiple copies of a particular RNA or DNA segment from a sample so that enough exists to be detected and identified.

### Multiplexing Speeds the Process

“Traditionally, the USDA labs look for one disease at a time,” says Lenhoff. Separate tests are made for each disease, using a variety of laboratory techniques. If the reading is negative for FMD, the test does not identify the disease agent that is present. To speed diagnosis, the Livermore team developed a multiplexed assay—one that simultaneously tests a single sample for all similar diseases, both endemic and foreign. Thus, a negative reading for FMD will likely be accompanied by a positive response identifying a similar disease. “With our technology, laboratories will be able to screen for many diseases at one time,” says Lenhoff. “In this way, our

assay provides more confidence in the negative FMD diagnosis.”

Multiplexing reduces labor and the costs of reagents. Compared with traditional diagnostic tests, the Livermore assay significantly increases speed and convenience of testing. The assay screens for both DNA and RNA viruses at the same time and detects the seven major subtypes of FMD. The test looks for 17 target signatures, including 2 that detect all 7 serotypes, or strains, of FMD. In all, the 17 signatures represent 7 diseases. Three of these are the foreign diseases FMD, vesicular exanthema of swine, and swine vesicular disease. Four are the endemic diseases bovine viral diarrhea, bluetongue, bovine herpes-1 (also known as infectious bovine rhinotracheitis), and bovine parapox virus complex (bovine papular stomatitis, contagious ecthyma, and pseudocowpox). The panel also incorporates five internal controls to increase confidence in the results.

The assay uses multiplex analysis technology developed by Bio-Rad Laboratories, a bead-based liquid array technology by Luminex Corporation, and components supplied by other companies. The assay first extracts nucleic acids from the sample and purifies them. RNA from RNA viruses is then converted to DNA, and all DNA is amplified.

(a)



To test the Laboratory's multiplexed assay, (a) analytical chemist Ben Hindson collected samples at a dairy farm near Davis, California, and (b) veterinarian Pam Hullinger collected them at the animal science facilities of the University of California at Davis.

(b)



Luminex polystyrene beads are individually tagged with a sequence of nucleotides that complement the signatures of interest. The beads are embedded with precise ratios of two fluorescent dyes, each with a unique spectral address. Each microbead is then interrogated by a flow cytometer. A laser excites the dye molecules inside a microbead to indicate the kind of microbead—that is, the pathogen—it is screening. Another type of laser excites fluorescently labeled molecules bound to each microbead's surface during the assay, which indicate how much of the pathogen is present in the sample. The flow cytometer can read up to 400 beads per second and simultaneously test for 100 different pathogens in a single sample.

### Extensive Performance Evaluation

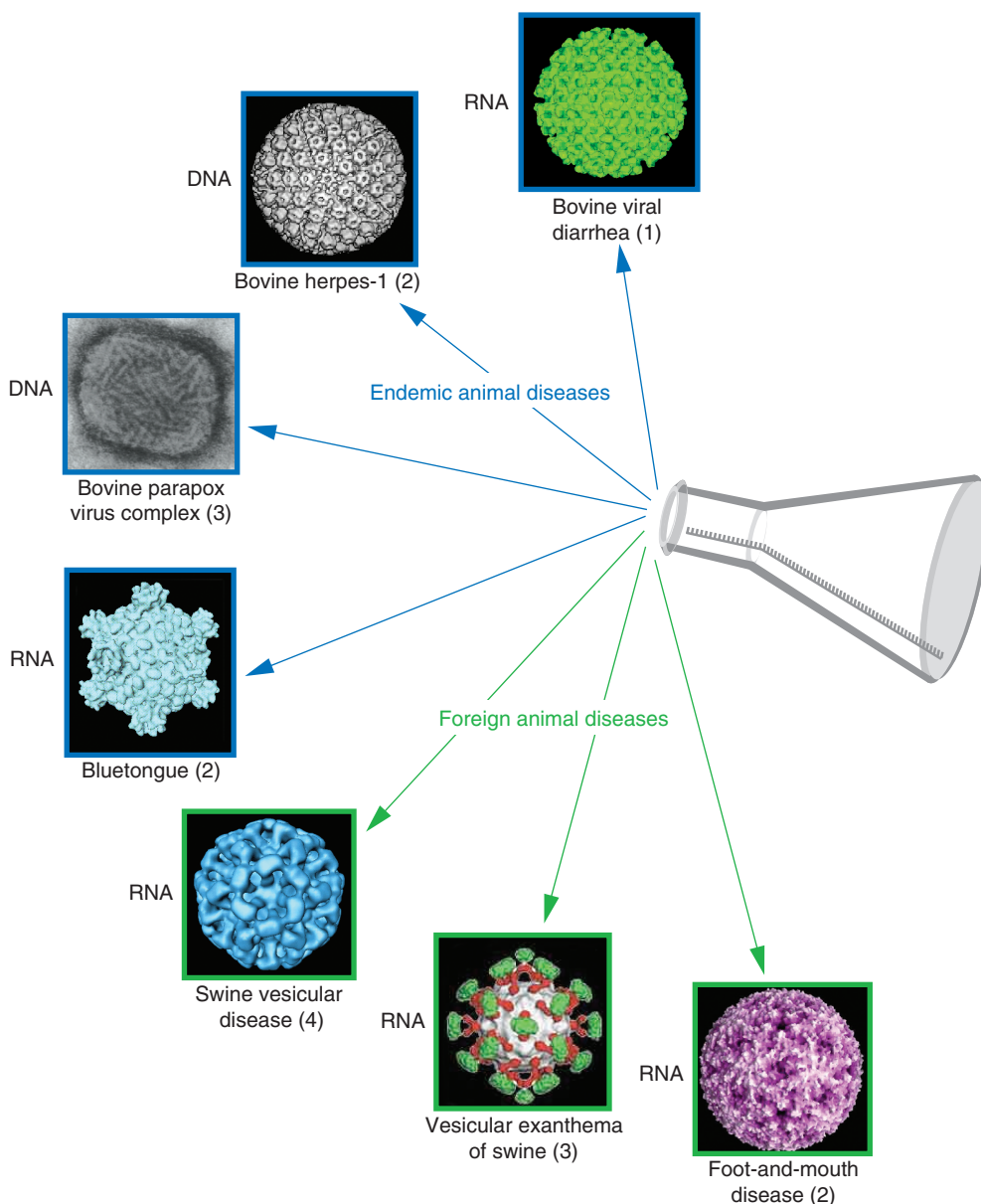
The Livermore assay is undergoing thorough testing, which consists of an NAHLN interlaboratory comparison and follow-on evaluation. During October and November 2005, 13 NAHLN laboratories and NVSL were equipped with the necessary tools for performing the assay, including a specialized flow cytometer (Bio-Rad Bio-Plex Workstation), small disposable items such as pipette tips and plastic ware, and reagents. The equipment became the property of the participating laboratories. Livermore personnel worked with Bio-Rad field service engineers and application specialists to conduct training at each NAHLN laboratory. The Livermore training team, which included Rasmussen, Christina Sanders, Jaqui Lee, James Thissen, and Celena Carrillo, gave instructions on operating the workstation, performing the assays, and analyzing and interpreting data.

Each laboratory then analyzed nearly 200 clinical samples “spiked” with known domestic viruses that mimic FMD. The samples were sent from Livermore in a viral transport medium that preserves the virus. “All of the laboratories ran the same tests on the new equipment using our assays and reported the results back to us,”

says Hindson, who oversaw training and equipment installation.

During the follow-on evaluation this year, NAHLN laboratories will analyze clinical diagnostic samples they receive from the field using their standard assays and Livermore's multiplexed assay. Results

from the two analyses will then be compared. “The labs have reference tests that are not necessarily based on PCR,” says Hindson, who is also coordinating the interlaboratory comparison. “The tests could be based on immunology, fluorescent staining, or scanning electron microscopy. Comparing



The Livermore assay tests for three foreign animal diseases (green border) and four endemic diseases (blue border). Electron micrographs of each virus are shown. Numbers in parentheses denote the number of signatures the assay uses to detect each disease.



A scientist at Indiana Animal Disease Diagnostic Laboratory at Purdue University learns to use the Livermore multiplexed assay.

results from the two methods will help us characterize the performance of our assay and establish an even greater level of confidence in its results. It also allows us to evaluate the assay's performance in the hands of the end users."

### High Throughput Using Robots

The team has also developed a high-throughput, semi-automated system that uses two robotic workstations. Together, the workstations can analyze 1,000 samples in a 10-hour shift with two laboratory technicians. "If there is a disease outbreak, we need a high-throughput capability to process a large number of samples," says Lenhoff.

Two NAHLN laboratories are testing the robotic system in a seven-day exercise this spring to simulate an outbreak scenario.

The demonstrations include field sample collection and identification, sample receipt, sample processing, data analysis, and a mechanism for reporting results.

The high-throughput system integrates the USDA's electronic sample identification technology with the laboratories' information management systems. "We want to demonstrate to state and federal authorities and laboratory personnel an integrated system that can function end to end during the response to a FAD outbreak, from collecting and processing samples through reporting the results," Hindson says. The portable system can be deployed to any location. The team is also developing a laboratory mounted inside a truck, which could provide mobile regional diagnostic support in the early phases of an outbreak response.

## Calculating the Cost of Foot-and-Mouth Disease

The foot-and-mouth disease (FMD) virus consists of a single strand of RNA packed inside a tough protein coat. The hardy virus can live 28 days in cool soil and up to 180 days in a slurry of cow dung. It can be spread directly from animal to animal, or it can be transported indirectly by a person or vehicle traveling from one farm to another—for example, with mud containing the virus caked on boots or tires. Livermore's Pam Hullinger, a foreign animal disease diagnostician and veterinary epidemiologist, notes that FMD is so contagious, an animal can become infected after contact with as few as 10 virus particles.

Although the U.S. has not had an FMD outbreak since 1929, it is endemic in many parts of the world. In 2000 and 2001, a Pan-Asian strain caused outbreaks in Korea, Japan, Russia, Mongolia, South Africa, the United Kingdom, France, and the Netherlands. Economic losses from an agroterrorist incident, especially one caused by an extremely contagious disease such as FMD, could be enormous.

The 2001 outbreak of FMD in the United Kingdom is estimated to have caused about \$5 billion in losses to the food and agriculture sector and even greater losses in tourism. Up to 10-million sheep, pigs, and cows were slaughtered, and for several months, the nation was banned from exporting livestock and animal products that could potentially transmit the virus.

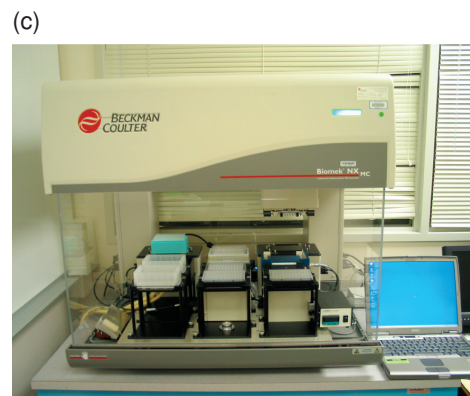
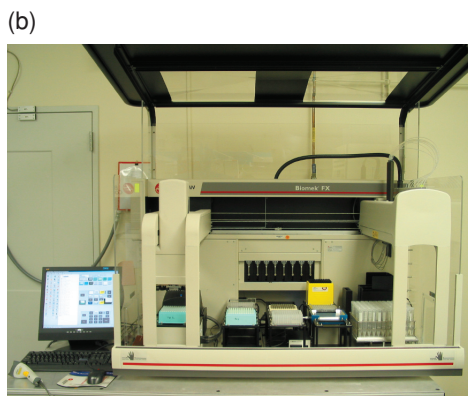
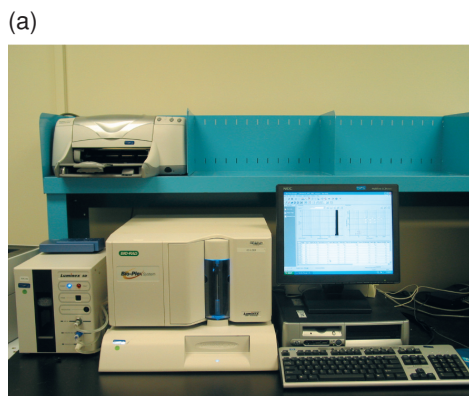
Hullinger answered the call for expert veterinarians during the 2001 epidemic and saw many animals slaughtered. "There is no substitute for first-hand experience in gaining respect for the devastation that a foreign animal disease can inflict on a nation,"

she says. Many veterinarians were asked to decide whether a flock or herd was infected based solely on clinical signs observed during an initial visit without the aid of testing or laboratory support.

"We now know that many uninfected animals were destroyed because we lacked rapid diagnostics for FMD," says Hullinger. "I saw thousands of animals killed in Britain, and thousands of farmers lost their livelihoods. I don't want to see that happen here."



Up to 10-million sheep, pigs, and cows were slaughtered during the 2001 outbreak of foot-and-mouth disease in the United Kingdom.



(a) A specialized flow cytometer, the Bio-Rad Bio-Plex Workstation, analyzes DNA using bead-based liquid array technology by Luminex Corporation. (b, c) To speed diagnosis during a disease outbreak, Livermore researchers designed robotic equipment to replace most manual operations.

## Looking Ahead

Later this year, APHIS will examine the results of this extensive evaluation and determine whether the multiplexed assay will be permanently adopted nationwide. In the meantime, the Livermore team meets regularly with USDA officials to better understand their needs. Although the assay was developed with a focus on agroterrorism, it could also help federal and state agencies perform routine agricultural disease surveillance. If a FAD were suspected based on a surveillance screening, then a disease investigation could be initiated quickly and samples sent to Plum Island to confirm the diagnosis.

Because the Luminex bead technology can screen for 100 different signatures at a time, the Livermore assay has room to expand. The team plans to add diseases to the original assay and develop new assays. One possibility is a multiplexed panel focused on diagnosing bovine fetal abortion, which can be caused by both viruses and bacteria. “We also want to develop species-specific assays for pigs, cows, and sheep,” says Lenhoff. Plans are already under way to develop assays for poultry.

Livermore chemist Julie Perkins is working with the National Center for

Foreign Animal Diseases in Canada to develop multiplexed assays that would determine both the type of FMD and whether animals vaccinated for FMD have also been infected by the virus. “Having a diagnostic tool available to rapidly and reliably determine if vaccinated animals have subsequently become infected is critical to the decision-making process involved in FMD vaccine use in the face of an outbreak,” says Hullinger.

Hullinger is also part of a Livermore effort to model FMD and other FADs so experts can evaluate different response scenarios to an outbreak. “We’re trying to give decision makers a better understanding of the effects of various disease control options,” she says.

“The labs are really excited,” says Hindson. “They can see a lot of value in the technology. For many applications, our multiplex technology can significantly improve their diagnostic capabilities.”

The Livermore team is just beginning to develop improved detection methods for animal diseases. National security and America’s agricultural industries are the clear winners.

—Arnie Heller

**Key Words:** agroterrorism, bioassay, foot-and-mouth disease (FMD), foreign animal disease (FAD), homeland security, National Animal Health Laboratory Network (NAHLN), National Veterinary Services Laboratory (NVSL), U.S. Department of Agriculture (USDA).

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